Growing blood vessels to treat limb ischemia: studie in mice and man
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Summary and Conclusions
Summary

This thesis describes the efforts of increasing our knowledge and insights into cellular and molecular mechanisms of vascular growth, especially collateral artery growth (arteriogenesis), in limb ischemia with the aim of developing new strategies for therapeutic angiogenesis and arteriogenesis. Conclusions are drawn from experiences in a mouse model mimicking peripheral artery disease, biopsies from human amputated limbs, and a trial using angiogenic gene therapy in patients.

Chapter 1 reviews what is known to date about the cellular and molecular mechanisms of vascular growth, with particular emphasis on the role of angiogenic factors, the immune system, and bone marrow. Furthermore, results and limitations of previous therapeutic angiogenesis trials for treatment of peripheral artery disease are reviewed. In conclusion, disappointing results from recent placebo-controlled trials merit more basic research in this field, with focus on optimizing cell-based therapies.

The large variety of surgical techniques and end point measurements that are applied between studies using ischemic hind limb models are discussed in Chapter 2. A guideline is provided on how to interpret results from these studies depending on the model used. In this thesis, a mouse model was selected to study the development of collateral arteries by selecting an appropriate surgical approach, using detailed angiography to visualize collaterals and determining their hemodynamic significance by perfusion measurement.

In Chapter 3, the unanticipated finding is described that mouse strains with different bias in immune responsiveness show major differences in collateral forming capacity. Subsequently, it was hypothesized that lymphocytes play a role in collateral formation. Arteriogenesis was impaired in mice lacking natural killer (NK) cells, however not in mice lacking natural killer T (NKT)-cells (a regulatory subset of T-cells). Furthermore, arteriogenesis was impaired in mice lacking CD4+ T-cells. This impairment was even more profound if these mice were depleted of NK-cells. In addition, evidence was provided that T-cells and NK-cells accumulate around collateral arteries, and secrete a variety of inflammatory cytokines. In conclusion, these data show that NK-cells and CD4+ T-cells are involved in collateral formation in mice. Stimulation of arteriogenesis by specific activation of defined lymphocyte subsets might be a promising treatment for patients with ischemic disease.

Chapter 4 provides evidence in human ischemic skeletal muscle for a role of vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) in adult neovascularization via retention of CXCR4-positive cells. Moreover, VEGF, SDF-1 and CXCR4 expressions in ischemic muscle were up-regulated in 2 patients
with acute-on-chronic ischemia, whereas down-regulated in 9 of 13 patients with chronic ischemia. This may be explained by an inability of hypoxic tissues to sufficiently express the transcription factor hypoxia inducible factor 1α (HIF-1α) in chronic ischemia. These data are the first to show in humans a pivotal role of SDF-1 in the retention of bone marrow-derived cells in hypoxic tissues. Furthermore, future experiments aiming on differences in angiogenic expression profile between acute and chronic hypoxic conditions may lead to optimized angiogenic treatments for patients with chronic ischemic disease.

In Chapter 5, it was shown that there was no impairment of angiographic collateral formation and only limited impairment of perfusion recovery in diabetic or insulin-resistant mice. Collateral formation was, however, severely impaired in hypercholesterolemic mice fed on high-fat diets. There was an inverse correlation of perfusion recovery with plasma cholesterol levels, but not with triglyceride, free fatty acid, glucose or insulin levels. In conclusion, impairment of arteriogenesis is more associated with hyperlipidemia than hyperglycemia or hyperinsulinemia, and is cholesterol-dependent in mice. In line with this, evidence is accumulating that a disturbed lipid metabolism is a crucial determinant of the development of diabetes and its complications. Therefore, a disturbed lipid profile might be crucial for the impairment of collateral formation in diabetic patients, stressing the importance of lipid-lowering drugs to prevent complications of diabetes.

In Chapter 6, it was demonstrated that VEGF gene therapy results in enhanced expression of myoglobin, a protein that plays an important role in oxygen metabolism of muscle cells, in ischemic skeletal muscle in mice. Furthermore, we show co-expression of VEGF and myoglobin in muscle biopsies from patients after limb amputation caused by peripheral arterial disease, which correlates with the degree of ischemia. In addition, a direct regulation of myoglobin by VEGF was shown in murine myotubes in culture. Our data indicate that VEGF therapy, apart from inducing new capillaries, changes properties of skeletal muscle fibers resulting in improved muscle oxygenation, which may explain the puzzling inconsistencies shown in previous clinical trials with VEGF. VEGF-mediated increase of muscle myoglobin may clarify, at least partly, the observed clinical improvements of VEGF-treated patients in the absence of improved vascular status.

Chapter 7 consists of the results from a double-blind randomized trial comparing intramuscular VEGF165 plasmid treatment (N=27) with placebo (N=27) in diabetic patients with critical limb ischemia. The primary end point was the amputation rate at 100 days. Secondary end points were a 15% increase in pressure indices between ankle and arm, clinical improvement and safety. In VEGF- versus placebo-treated patients, amputation occurred in 3 versus 6 (NS), hemodynamics improved in 7 versus 1 (P=0.05), skin ulcers healed in 7 versus 0 (P=0.01), and pain decreased in
5 versus 2 (NS). No substantial adverse effects were observed. Although this trial failed to meet the primary objective of significant amputation reduction, VEGF gene therapy resulted in some significant clinical improvements. Hopefully, this study could serve to regenerate interest in therapeutic angiogenesis after recent disappointing trials.

Conclusions

In this thesis a mouse model was used to study the cellular and molecular mechanisms of arteriogenesis. Only recently, one has begun to unravel the role of the immune system and its cellular components in collateral artery growth. Here, evidence is provided for a role of lymphocytes. More insights into which (other) cell types are involved will enlarge the toolbox for stimulation of arteriogenesis and may refine autologous bone-marrow transplantation, as recently applied in clinics, for instance by administration of defined lymphocyte subsets or their specific activation/inhibition with ligands for activating or inhibitory receptors, respectively. Using the mouse model, knowledge may not only be brought from bench to bedside, but also from bedside to bench. For instance, the unexplained beneficial results in VEGF-treated patients without improved vascularization led to the hypothesis that VEGF may not only simply "grow vessels", but may also improve muscle oxygenation by changing muscle composition, which was then proven in mice. This finding in turn holds considerable promise for the development of novel therapeutics to treat various diseases. Nevertheless, it is important to realize that mice are not patients. Although genetically modified mice mimic disease profiles of patients, for instance by inducing dyslipidemia or diabetes, vascular disease in patients seems more complex; multiple vascular risk factors coexist, and endothelium, bone marrow and/or the immune system may be dysfunctional, leading to impaired arteriogenesis. Moreover, in this thesis evidence is provided for an inability of hypoxic tissues to express angiogenic factors in patients with chronic ischemia. More insights into impaired vascular growth in patients are important, since most placebo-controlled trials with angiogenic or arteriogenic factors were negative to date. Various reasons, ranging from the type of angiogenic factor to technique of administration to patient selection may account for this. The design of the trial described in this thesis using intramuscular plasmid VEGF with some beneficial effects in diabetic patients with critical limb ischemia, together with the novel mechanistic insights from the mouse studies, may provide a handhold for the development of new trial protocols. Finally, progress in the research field of arteriogenesis may not only prove beneficial for the treatment of peripheral artery disease, which is the aim of this dissertation, but also of coronary heart disease, the leading cause of death to date. One of many challenges is to elucidate similarities and differences in cellular and molecular mechanisms of vascular growth between limb and heart, which may help to extrapolate results from clinical trials conducted in the field of vascular surgery towards the field of cardiology and vice versa.